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Gene Amplification as a Developmental Strategy: Isolation of Two Developmental Amplicons in *Drosophila*

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Summary

Gene amplification is known to be critical for upregulating gene expression in a few cases, but the extent to which amplification is utilized in the development of diverse organisms remains unknown. By quantifying genomic DNA hybridization to microarrays to assay gene copy number, we identified two additional developmental amplicons in the follicle cells of the *Drosophila* ovary. Both amplicons contain genes which, following their amplification, are expressed in the follicle cells, and the expression of three of these genes becomes restricted to specialized follicle cells late in differentiation. Genetic analysis establishes that at least one of these genes, *yellow-g*, is critical for follicle cell function, because mutations in *yellow-g* disrupt eggshell integrity. Thus, during follicle cell differentiation the entire genome is overreplicated as the cells become polyploid, and subsequently specific genomic intervals are overreplicated to facilitate gene expression.

Introduction

Some tissues require the production of massive amounts of particular gene products during periods of development so brief that increased transcription alone is insufficient. One mechanism by which sufficient gene expression can be achieved is via amplification of the genes prior to their transcription, leading to an increase in the amount of template available for transcription. Such developmentally regulated gene amplification is employed for the ribosomal RNA genes in amphibian oocytes to facilitate stockpiling of the oocyte with ribosomes (Brown and Dawid, 1968; Gall, 1968), as well as during the production of the macronucleus in Tetrahymena (for review see Prescott, 1994). In the Sciarid flies, the salivary gland rapidly synthesizes structural proteins for cocoons by amplifying these genes (Glover et al., 1982; Rudkin and Corlette, 1957; Wu et al., 1993). In the follicle cells of *Drosophila melanogaster*, the genes for the structural proteins of the eggshell (chorion) are amplified prior to their transcription (Spradling, 1981). In the latter case, amplification is essential for adequate levels of gene expression because mutations that reduce amplification cause thin eggshells and female sterility (Orr et al., 1984).

Genomic technologies now provide the opportunity to determine the global use of gene amplification during development. The last developmental amplicon was identified over 20 years ago. Early identification of amplified DNA relied on cytological evidence such as DNA puffs in the case of the Sciarid flies or extra chromosomal DNA circles in oocytes. Subsequently, amplicons were identified by testing DNA clones encoding developmentally expressed genes for increased gene copy number during differentiation, but this methodology was employed only sporadically (for review see Spradling and Orr-Weaver, 1987).

The developmental requirement for chorion gene amplification and its role in follicle cell differentiation are understood (for review see Orr-Weaver, 1991). The genes encoding six of the major structural components of the eggshell are clustered on the X chromosome at cytological location 7F and on the third chromosome at 66D. The follicle cells first synthesize and secrete the vitelline membrane proteins onto the oocyte surface, then secrete the chorion proteins to build a multilayered eggshell. Prior to the transcription of the major chorion protein genes, the genomic intervals containing these genes are amplified to increase the amount of template available for transcription. The genes encoding the vitelline membrane proteins as well as those encoding other minor chorion proteins, however, are not amplified in the follicle cells (Higgins et al., 1984; Popodi et al., 1988). Instead, they are transcribed over a longer period of approximately 15 hr (Mahowald and Kambysellis, 1980), as compared with the major chorion protein genes that must be transcribed over approximately 2 to 3 hr (Parks and Spradling, 1987).

In addition to being an intriguing developmental paradigm, analysis of insect amplicons has provided key insights into the regulation of metazoan DNA replication. At these sites, gene amplification occurs by repeated firing of replication origins within the gene clusters and movement of replication forks to produce a gradient of amplified DNA. In *Drosophila*, *cis*-acting control elements have been delineated by transformation experiments (Carminati et al., 1992; de Cicco and Spradling, 1984; Lu et al., 2001), and in both *Drosophila* and Sciara the positions of the replication origins used in amplification have been mapped (Bielinsky et al., 2001; Delidakis and Kafatos, 1989; Heck and Spradling, 1990). Initiation factors and proteins utilized for normal genomic replication are essential for amplification (for review see Bosco and Orr-Weaver, 2002). In fact, the Origin Recognition Complex (ORC) was first demonstrated to bind specific metazoan genomic sequences at the third chromosome chorion amplicon (Austin et al., 1999). ORC also binds to key replication elements in the Sciara amplicon (Bielinsky et al., 2001). Mutations that disrupt amplification have led to the identification of new replication factors (Landis and Tower, 1999; Whittaker et al., 2000). In *Drosophila* follicle cells, all of the replication initiation events

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for the chorion amplicons occur in two specific stages of egg chamber development. During subsequent stages, replication forks elongate in the absence of additional initiation events (Claycomb et al., 2002). This developmental separation of initiation and elongation permits distinct roles for proteins in these two processes to be distinguished.

The adaptation of microarrays to measure gene copy number provides a means to screen for gene amplification events across the genome throughout development. The recovery of additional amplicons provides model replicons and serves as a means to identify genes whose functions are crucial at particular developmental points. It is of interest to learn what types of proteins, in addition to ribosomal proteins or structural proteins of the eggshell or cocoon, require amplification in some contexts. Moreover, given the role that amplification of oncogenes plays in cell transformation (Gray and Collins, 2000), it is conceivable that amplification of cell division or tumor suppressor genes could regulate replication or division during differentiation. Here, we use microarrays to identify two follicle cell amplicons and demonstrate that these encode genes are expressed in and essential for follicle cell function.

Results

Identification of Amplified Genes on Microarrays

We developed a technique to identify sites of DNA amplification by copy number comparison using microarrays that were simultaneously hybridized with experimental genomic DNA labeled with one fluorochrome and control genomic DNA labeled with another. We focused our studies on gene-encoding regions of the genome by producing microarrays of cDNAs from the *Drosophila* Gene Collection. This collection contains unique full-length cDNAs for 5928 genes and represents 42% of the predicted protein-coding genes in the *Drosophila* genome (Rubin et al., 2000). In the two known follicle cell amplicons, the peak amplification levels are at the sites of the chorion genes, but there are gradients of increased DNA copy number extending about 50 kb to either side (Spradling, 1981). Thus we reasoned that, based on the average gene density in *Drosophila*, we were likely to detect genes within an amplified domain, even if all of the predicted genes were not present on the microarrays. The cDNA inserts for each clone were PCR amplified, and the PCR products were spotted onto slides to generate the arrays. The arrays were simultaneously hybridized with Cy-5-labeled control genomic DNA from 0–2 hr (2C) embryos and Cy-3 labeled genomic DNA from FACS-sorted 16C follicle cell nuclei. The follicle cells become polyploid before undergoing chorion gene amplification; thus the 16C population is enriched for amplified DNA.

The ratio of the hybridization signal between the follicle cell genomic DNA probe and the control embryonic genomic DNA probe revealed increases in copy number of specific genes in follicle cells. The experiment was repeated three times to ensure reproducibility. As a positive control, the array included five clones from the third chromosome chorion amplicon and three from the *X* amplicon. These genes flank the maximally amplified regions containing the chorion genes, and most displayed copy number increases of greater than 2-fold in our assay (Table 1). Nine of the other clones that were significantly amplified (see Experimental Procedures) were striking because six were localized together within 65 kb at cytological interval 30B10, and three were clustered within 10 kb at 62D5 (Table 1) (Figures 1C and 1E). In addition to these eight clones, other genes within the 30B and 62D regions had increased copy numbers close to the significance cut off (Table 1). These observations strongly suggested that the microarray experiments had identified two additional follicle cell amplicons, and we named them DAFC (Drosophila Amplicon in Follicle Cells)-30B and -62D. For consistency, we will refer to the chorion amplicons as DAFC-7F (X chromosome) and DAFC-66D (third chromosome).

Confirmation that DAFC-30B and DAFC-62D Are Follicle Cell Amplicons

To validate the microarray data and verify that DAFC-30B and 62D are amplified in the follicle cells during late oogenesis, we used the approaches of quantitative real-time PCR and fluorescent in situ hybridization (FISH) with Bromodeoxyuridine (BrdU) colabeling of the follicle cells. We have previously employed quantitative real-time PCR to determine DNA copy number across the amplified domains DAFC-66D and -7F (Claycomb et al., 2002, and data not shown). Genomic DNA was isolated from distinct populations of egg chambers: (1) stage 1–8 egg chambers, developmental stages prior to the onset of gene amplification in the follicle cells at stage 9; (2) stage 13 egg chambers at the peak of gene amplification; or (3) 16C amplifying follicle cell nuclei (as described above). These genomic DNAs were used as templates for primers spaced at 5 kb intervals along the putative amplification domain. We observed reproducibly that the 30B and 62D genomic intervals were amplified, with copy number increases extending across 75 kb for DAFC-62D and 100 kb for DAFC-30B (Figures 1A and 1D).

DAFC-62D is amplified a maximum of 6-fold at an intergenic region, a lower level than the 14-fold amplification at DAFC-7F and 30-fold at DAFC-66D (Claycomb et al., 2002, and data not shown). The peak of amplification in the DAFC-62D gradient suggests the position of an origin of DNA replication. To map this peak more precisely, we performed real-time PCR in 2 kb intervals at the maximally amplified region. This confirmed the peak to be approximately 1.5 kb from the 3′ side of the *yellow-g2* gene (Figure 1B). We also investigated the developmental timing of replication initiation at the amplification peak by measuring copy number changes in stage 10B, 11, 12, and 13 egg chambers. We showed previously that DAFC-7F and 66D completed initiation by stage 11 and in subsequent stages existing replication forks elongated (Claycomb et al., 2002, and data not shown). In contrast, DAFC-62D undergoes a late round of initiation between stages 12 and 13 (Figure 1B). It appears that the forks from this last initiation do not progress far, resulting in a small region of increased amplification in stage 13 egg chambers (model inset, Figure 1A).

The peak levels of amplification for DAFC-30B are 4-fold, and the maximum copy number increase is distributed over a 50 kb region (Figure 1D). We examined

Table 1. Microarray Experiments Reveal Clusters of Genes with Increased Copy Number

Gene (Clone ID)	Fold Amplified Trial 1	Fold Amplified Trial 2	Fold Amplified Trial 3	Gene Function/Homology
DAFC-66D				
srpRβ (GM04779)	16.56	10.33	17.63	Signal Recognition Particle Receptor-β
prm (GH17893)	21.16	13.93	14.90	Paramyosin
prm (GH14085)	17.45	16.08	15.08	
CG32030 (LD24110)	2.64	4.28	2.50	Actin binding
CG32030 (SD08909)	ND	2.66	3.96	
DAFC-7F				
sptr (GH04031)	5.47	4.68	7.90	Sepiapterin Reductase
es2 (SD03464)	ND	2.93	6.33	Sepiapterin Reductase
CG12123 (GH02722)	1.34	ND	2.51	Novel
CG1440 (LD46760)	ND	2.13	2.47	Cysteine-type Endopeptidase
DAFC-62D				
CG1275 (LD36721)	1.59	1.40	1.73	Vesicle Electron Transporter
oxt (LD43716)	ND	ND	2.29	Glycosyl Transferase; Core-2/I-Branching Enzyme
CG5714 (GH14368)	3.11	1.84	1.25	Novel
CG32302 (LP11057)	1.73	2.54	1.98	Chitin Binding
DAFC-30B				
CG3811 (GH04717)	2.30	3.18	3.18	Transporter; Kazal-type Serine Protease Inhibitor
CG31883 (GH13755)	2.72	2.61	ND	Chitin Binding
Gdi (LD46767)	1.35	0.88	1.52	GDP-dissociation Inhibitor; Synaptic Vesicle Fusion
CG3838 (LD04047)	1.57	1.73	1.09	Novel
CG3838 (LD21447)	ND	ND	1.93	
CG4389 (GH12558)	1.54	1.85	1.56	Long Chain Enoyl-CoA Hydratase
CG18419 (GM07803)	2.18	2.12	2.36	Ca2+ Transporting ATPase
jp (GH28348)	ND	1.93	1.67	Junctophilin Matrix Protein

Microarray experiments were repeated three times. Clones flanking the known chorion amplicons at DAFC-66D and -7F were positive controls. Threshold for significance is described in Experimental Procedures. ND, not determined. Gene homologies or predicted functions are as listed in Flybase or determined by BLAST.

the developmental timing of initiation at this amplicon but found that the initiation events were completed by stage 10B (data not shown). Thus the breadth of the amplification peak most likely results from elongation of these forks during stages 11–13 without additional rounds of initiation (model inset, Figure 1D), an amplification profile similar to that of DAFC-66D and -7F.

As a second approach to confirm that DAFC-30B and -62D were amplicons, we directly observed replication patterns in follicle cells. The presence of two additional amplicons in follicle cells was suggested by the pattern of BrdU labeling during amplification stages (Calvi et al., 1998). At developmental times when genomic replication has ceased, follicle cells show BrdU incorporation in four foci. The larger two foci were shown by FISH to be the chorion gene clusters, but the identity of the smaller two foci remained unknown (Calvi et al., 1998). To test if DAFC-30B and DAFC-62D were replicated during amplification stages, we performed double labeling with FISH and BrdU. The FISH probe for each amplicon colocalized to one of the two small foci of BrdU incorporation in stage 10B follicle cells (Figure 2). The combined results of the microarray analysis, real-time PCR, and FISH-BrdU labeling experiments establish that DAFC-30B and -62D are two follicle cell amplicons.

Predicted Amplified Gene Products

Because our goal was to find additional examples of gene amplification that are necessary for proper development, we needed to determine the developmental relevance of the amplified genes in DAFC-30B and -62D. Our first step to evaluate the developmental significance of DAFC-30B and -62D amplification was to examine the homologies of the genes encoded in these regions. There are a variety of genes in DAFC-30B and -62D, none of which have been previously associated with mutant phenotypes or homologies that implicate them as functioning in oogenesis or eggshell formation. However, we found it notable that there were at least two groups of genes in the amplicons encoding proteins that could potentially function in egg production.

The maximally amplified genes in DAFC-62D, *yellow-g* and *yellow-g2*, are members of the *yellow* gene family that are predicted to encode secreted proteins (Drapeau, 2001; Maleszka and Kucharski, 2000). The family shares homology with the Major Royal Jelly Protein Family in honeybees (*Apis mellifera*), involved in the specification of the queen bee (Albert et al., 1999; Maleszka and Kucharski, 2000). The founding member of the Yellow family, Yellow-y, is known to play a role in mating behavior and in the melanization and hardening of the adult cuticle. Other Yellow family members have been shown to act as dopachrome-conversion enzymes that catalyze a key reaction in the melanization process (Han et al., 2002; Sugumaran, 2002). Interestingly, a similar process is used in the hardening of the egg chorion in mosquitoes (Li, 1994) and suggests that Yellow-g and Yellow-g2 may play a catalytic role in the crosslinking of the chorion and/or underlying vitelline membrane proteins in *Drosophila*.

A second group of genes encodes proteins with chitin binding motifs that could function in egg production. Genes of this type are present in both amplicons, with DAFC-62D containing two such genes and DAFC-30B

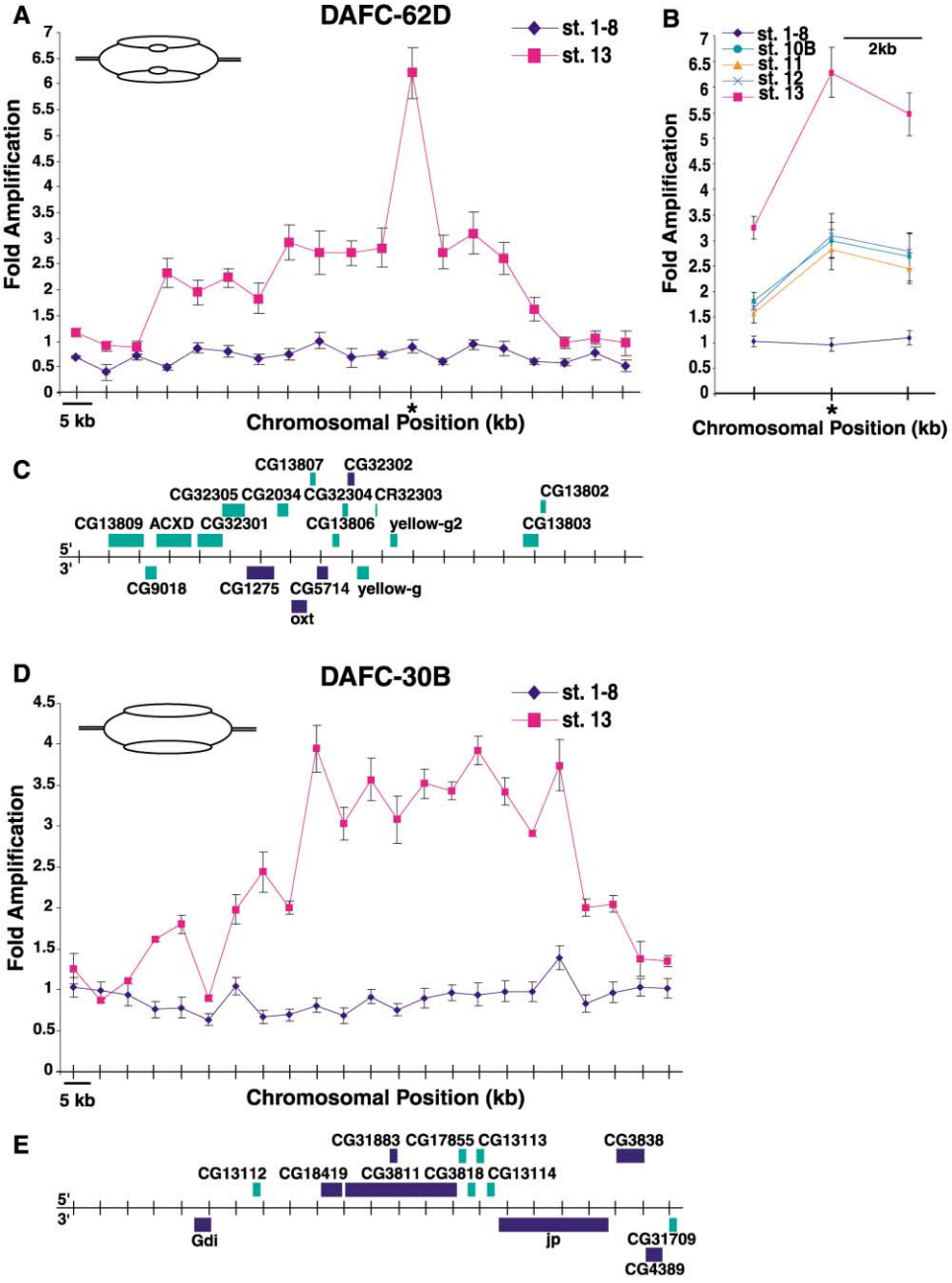

Figure 1. Real-Time PCR Demonstrates that the Genes in DAFC-62D and -30B Are Amplified during Late Oogenesis

In (A) and (D), the fold amplification of each region, relative to a nonamplified portion of the genome and to 2C embryo genomic DNA standards, was determined in 5 kb intervals by quantitative real-time PCR performed on preamplification- (st. 1–8, blue diamonds) and amplification- (st. 13, pink boxes) stage whole egg chamber genomic DNA. Error bars are the standard deviations of triplicate reactions (Claycomb et al., 2002). (B) The peak of amplification at DAFC-62D ([A], asterisk) was analyzed by quantitative real-time PCR in 2 kb intervals.

In (C) and (E), the locations of the genes in 5 kb intervals along the DAFC-62D and -30B amplicons, respectively, are diagrammed. Those genes represented on the microarray are shown in blue; others are in green.

Tick marks in (A) and (D) correspond to those in (C) and (E), and the fold amplification for each gene can be examined by tracing upward to the graph. The insets in (A) and (D) show models for the gradients of amplification.

containing one. Chitin binding domains serve an antimicrobial function in a variety of plants and marine invertebrates. Homologs of marine invertebrate proteins, such as tachycitin, could provide the egg with protection against microbes (Kawabata et al., 1996). Alternatively, chitin, a structural polysaccharide found in many organisms, could also be a component of the eggshell, and interaction with the chitin binding proteins might contribute to eggshell integrity.

In both DAFC-30B and 62D, there are also a number of genes whose role in follicle cells is not yet clear. These include both genes encoding proteins without known sequence motifs and genes whose products are predicted to have the enzymatic activities of adenylate

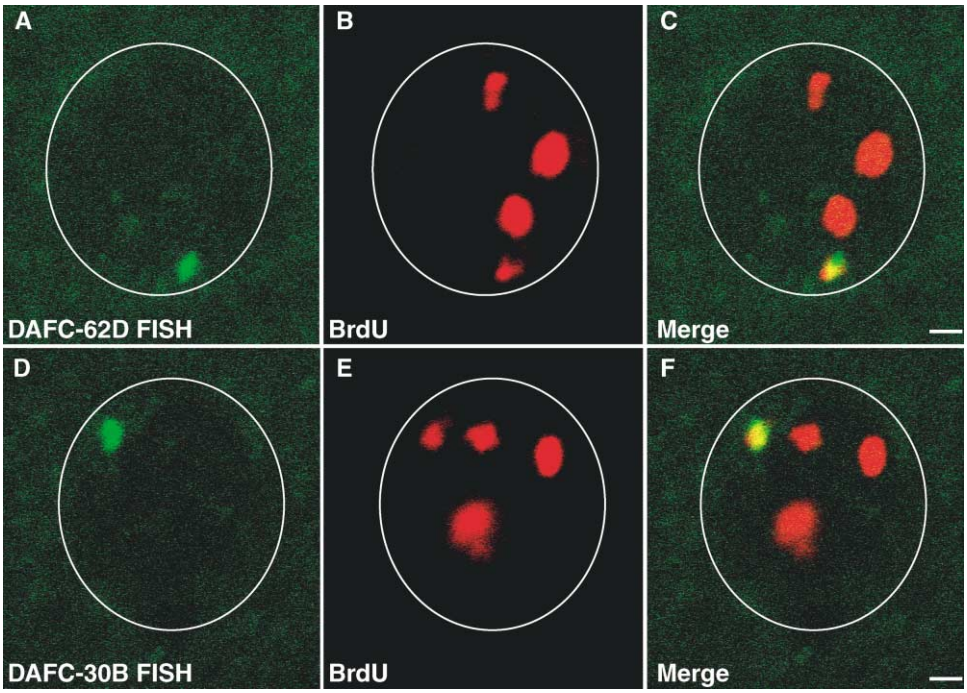

Figure 2. Fluorescent In Situ Hybridization and BrdU Labeling Reveals that DAFC-30B and -62D Correspond to Sites of Amplification in Follicle Cells during Late Oogenesis

A single representative follicle cell nucleus is shown, probed with a 10 kb fragment from DAFC-62D ([A], green), or with DAFC-30B ([D], green), and labeled with BrdU ([B]; [E], red). Colocalization is yellow in (C) and (F). The larger BrdU spots in each nucleus correspond to DAFC-7F and -66D. The scale bar represents 1 μm.

cyclases, membrane transporters, calcium-transporting ATPases, GTP dissociation inhibitors, and others (Table 1).

Expression Patterns of the Amplified Genes

Gene homologies suggested it likely that at least some of the amplified genes would play a role in oogenesis or eggshell formation. Furthermore, we predicted that if amplification were required to achieve optimal levels of expression by these genes, they would be highly expressed in the follicle cells during late oogenesis, after amplification had initiated. To assess this hypothesis, we examined gene expression in the ovaries by RNA in situ hybridization.

The *yellow-g* and *yellow-g2* transcripts from DAFC-62D were initially detected in a subset of stage 10B follicle cells at the anterior end of the oocyte, concentrated at the dorsal side (Figures 3C and 3D). In egg chamber stages 11 and 12, all the follicle cells, except those around the nurse cells, robustly expressed the transcript (Figure 3E). In stage 13, expression was restricted to the follicle cells that produce the micropyle, a hollow tunnel in the eggshell through which the sperm enters (Figures 3F and 3G). The specificity of this final expression suggests a role for these gene products in vitelline membrane or eggshell formation.

Several other genes from the amplicons were expressed during follicle cell differentiation, when the vitelline membrane and eggshell are forming. The *CG13113* transcript from DAFC-30B, encoding a protein of unknown function, initiates expression in dorsal follicle cells in late stage 10B, and is expressed across the follicle cell layer until stage 13. Late stage 13 egg chambers display high expression in a subset of follicle cells surrounding and building the dorsal appendages, structures that allow for gas exchange and respiration of the embryo, and in the posterior follicle cells (Figures 3H–3J). The DAFC-30B transcript *CG18419*, with homology to a calcium-transporting ATPase, is expressed throughout the follicle cell layer, but at highest levels in the dorsal follicle cells from stage 10B throughout later stages (Figures 3K–3M). We also observed that, from stage 10 onward, the transcripts from the *CG3811*, *CG3818*, *CG13803*, and *CG5714* genes are present in the nurse cells and at low levels throughout the follicle cell layer (data not shown).

yellow-g Is Essential for Proper Eggshell Formation

Although the homologies and expression patterns of the amplified genes are consistent with a role in eggshell or vitelline membrane formation or oogenesis in general, we sought to evaluate directly the necessity for amplified genes in these processes. In particular, the expression pattern of *yellow-g* and *yellow-g2* suggested that these genes would play a role in overall eggshell formation, or perhaps in the formation of eggshell substructures, such as the micropyle. To evaluate whether *yellow-g* was essential for follicle cell function, we analyzed the phenotype caused by a P element transposon insertion that disrupts the *yellow-g* gene.

In the *EY01493* line, there is a P element inserted in the 3′ exon of *yellow-g* (Bellen, 2003; Spradling et al., 1999). This mutation disrupted *yellow-g* expression, whereas the expression of *yellow-g2* and *Cp-38* was

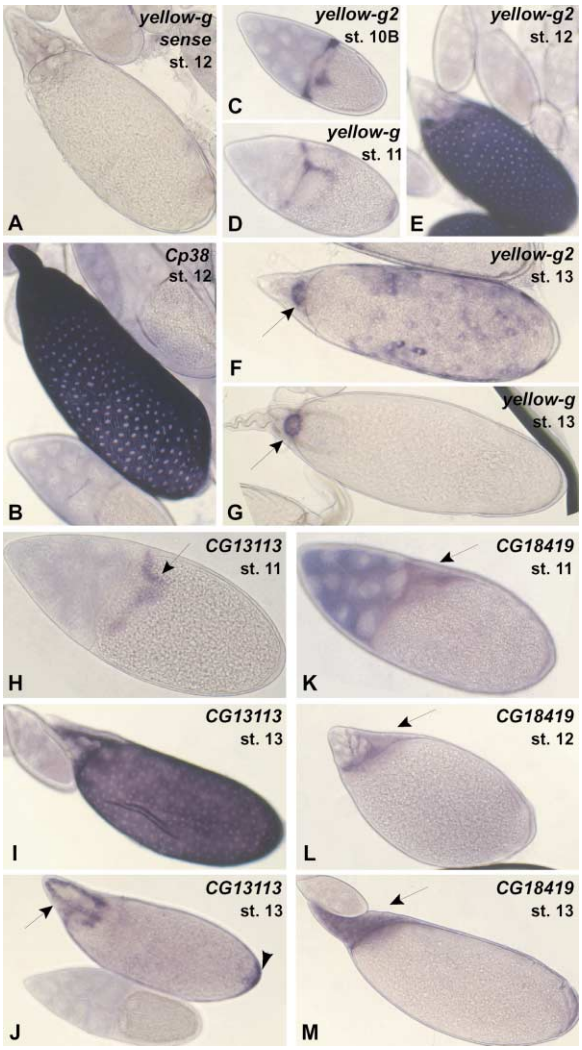

Figure 3. RNA In Situ Hybridization Shows that Genes in DAFC-62D and -30B Are Highly Expressed in Differentiating Follicle Cells

(A) The sense *yellow-g* probe shows no hybridization signal in any egg chamber stage. (B) The chorion gene *Cp38* is robustly expressed during egg chamber stages 11 and 12. *yellow-g2* (C) and *yellow-g* (D) initiate expression during late stage 10B, and transcripts accumulate over the egg chamber during stage 12 ([E], *yellow-g2* shown). During stage 13, expression decreases ([F], *yellow-g2*, slightly earlier stage 13 than in [G], *yellow-g*), and the mRNA is concentrated in the follicle cells around the micropyle (arrows). *CG13113* expression begins during late stage 10B ([H], arrow, stage 11 shown), and accumulates over the egg chamber in early stage 13 (I).

(J) In later stage 13 egg chambers, *CG13113* transcripts are restricted to the follicle cells covering dorsal appendages ([J], arrow), and at the posterior end ([J], arrowhead). *CG18419* is expressed in the anterior dorsal follicle cells beginning in stage 10B ([K], arrow, stage 11 shown), and into stage 12 ([L], arrow). In stage 13, *CG18419* mRNA accumulates over the entire anterior of the egg chamber, including the dorsal appendages ([M], arrow). Although it appears that *CG18419* is also expressed from the nurse cells in stage 11, we observed the same level of expression with the sense probe for this gene over the nurse cells in comparable stages of egg chambers (data not shown). Anterior is left.

unaffected (Figures 4A–4D). These mutant females were sterile, yet male fertility was not affected. Oogenesis proceeded normally in mutant females, but mature stage 14 oocytes often had indentations in the vitelline membrane, and at these sites the yolk was displaced (data not shown). Eggs laid by these mutant mothers were defective and collapsed, although the exochorion and dorsal appendages appeared normal (Figures 4E and 4F), indicative of defects in the vitelline membrane (Savant and Waring, 1989; Waring, 2000). These phenotypes show that *yellow-g* is needed for proper egg formation, possibly for the production of a structurally sound vitelline membrane, or to catalyze the crosslinking of eggshell layers for the rigidity of the egg.

Amplification Is Necessary for Gene Expression

Amplification of the chorion genes is required for high levels of expression, as mutations that disrupt DNA replication factor genes such as *double parked* (*dup/cdt1*), *origin recognition complex subunit 2* (*orc2*), *chiffon* (*chif*, *dbf4-like*), *proliferating cell nuclear antigen* (*pcna, mus209*), or *minichromosome maintenance factor 6 (mcm6)*, result in decreased amplification and thin eggshells (Henderson et al., 2000; Landis et al., 1997; Landis and Tower, 1999; Schwed et al., 2002; Underwood et al., 1990). These mutants also display decreased BrdU incorporation at the four amplified loci (Calvi et al., 1998; Schwed et al., 2002; Whittaker et al., 2000). To determine if amplification of DAFC-30B and 62D was necessary for adequate levels of gene expression, we performed RNA in situ hybridization to the *yellow-g, yellow-g2*, and *CG13113* transcripts in the *mcm6* and *chiffon* mutants. We found that transcript levels for all three amplified genes tested were reduced, but not eliminated, in the mutant ovaries (Figures 5A–5H).

Although *mcm6* and *chiffon* mutants have been reported to have decreased BrdU incorporation at the amplifying loci (Calvi et al., 1998; Schwed et al., 2002), we wanted to test directly whether DAFC-30B and -62D specifically were amplified in the mutants. Thus we performed FISH and BrdU colabeling on *mcm6* and *chiffon* mutant and sibling control ovaries. These experiments verified that DAFC-30B and -62D were not amplified to any significant degree, as could be detected by BrdU incorporation, in the majority of follicle cells (Figures 5I–5L). These data demonstrate that amplification of DAFC-30B and -62D is necessary for high levels of expression and reiterate that these amplicons rely on the normal replication machinery for their amplification.

Discussion

There are several mechanisms by which organisms can fulfill a need for bursts of gene expression, including carrying stable duplications of the highly expressed genes, upregulating transcription, upregulating translation, or by developmentally regulated amplification of specific genes. To date, only a handful of developmental amplicons have been examined, and the isolation of new amplicons has mainly relied on visual detection of amplified DNA. Thus it remains to be seen how widely gene amplification is used as a developmental strategy for robust gene expression across different species.

We have established a methodology for the systematic analysis of gene amplification as a developmental

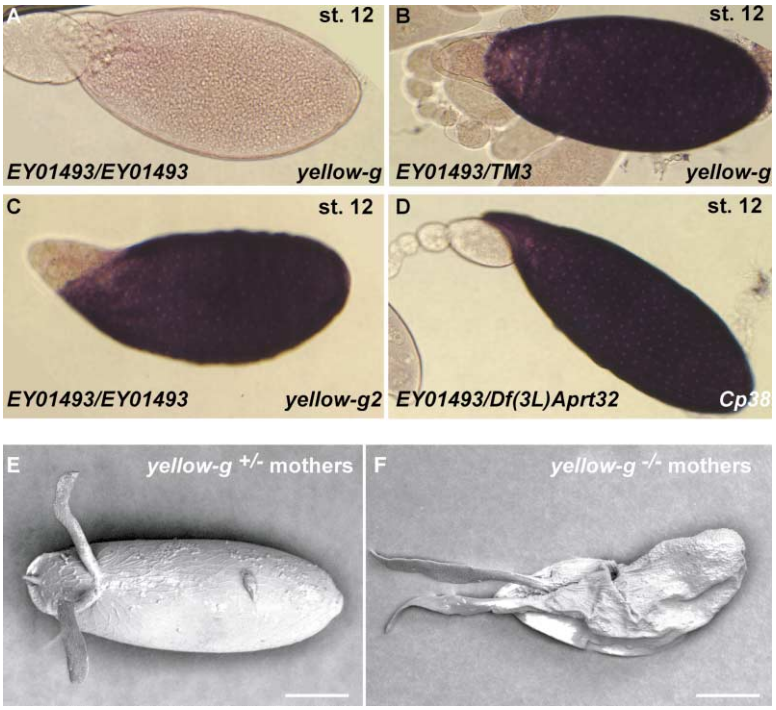

Figure 4. Characterization of the *yellow-g* Mutant

RNA in situ hybridization shows that *yellow-g* mutant females ([A], genotype: *EY01493/EY01493*) have decreased levels of the *yellow-g* transcript in comparison with control siblings ([B], genotype: *EY01493/TM3*). However, RNA in situ hybridization to mutant ovaries for *yellow-g2* (C) or *Cp38* (D) shows that females homozygous for the P element insertion *EY01493* ([C], genotype: *EY01493/EY01493*) or transheterozygous for the P element and the deficiency ([D], genotype: *EY01493/Df(3L)Aprt32*) display no changes in transcript levels. Anterior is left.

SEM was performed on embryos from mothers with a wild-type copy of *yellow-g* ([E], genotypes: *EY01493/TM6B* or *Df(3L)Aprt32/TM3*), or from *yellow-g* mutant mothers ([F], genotype: *EY01493/Df(3L)Aprt32*). Embryos from the *yellow-g* mutant mothers appear to have normal chorion and dorsal appendages, but eggs spontaneously collapse when laid. Anterior is left and dorsal is up. Scale bars are 100 μm, both images are magnified 180×.

strategy, and in doing so we have identified two additional developmentally regulated amplicons in the *Drosophila* follicle cells. The recovery of these amplicons validates the microarray approach to survey DNA copy number and provides additional model replicons to study. Additionally, the power of *Drosophila* genetics affords us a system to evaluate the functions of amplified genes in particular developmental processes. The process of amplification in the follicle cells reveals a progressive restriction of increased gene copy number in the genome. Initially, the entire euchromatin is increased in copy number as the follicle cells become polyploid. Later in follicle cell differentiation, only four specific genomic regions are amplified.

None of the amplified genes we identified in DAFC-30B and -62D had been previously implicated in eggshell formation, and thus recovery of additional amplicons also highlights developmental activities of the amplified genes. We showed that the *yellow-g* gene is essential for a rigid eggshell, and the predicted gene products of the *yellow-g* and *yellow-g2* genes suggest a molecular explanation for these mutant defects. The eggshell is composed of several layers, including the outermost exochorion, the endochorion, the inner chorion layer, and the vitelline membrane, which is the innermost structure that also contacts the oocyte (for reviews see Spradling, 1993, and Waring, 2000). The collapsed embryos and disrupted vitelline membranes that result from mutation of *yellow-g* indicate that *yellow-g* is necessary for the structural integrity of the eggshell. At the level of the light microscope, the exochorion of embryos laid by mutant mothers appears normal. The collapsed embryos are reminiscent of vitelline membrane defects (Savant and Waring, 1989), leading us to hypothesize that *yellow-g* is necessary for proper vitelline membrane formation.

We propose that Yellow-g and Yellow-g2 act to crosslink the vitelline membrane, or perhaps the inner chorion layer. The Yellow family members, Yellow-f and Yellow-f2, are capable of catalyzing the conversion of dopachrome to dihydroxyindole, a limiting step in the melanization pathway, during larval, pupal, and adult stages (Han et al., 2002). The enzymatic events leading to the crosslinking of the vitelline membrane are not well understood, but seem to involve one phase of disulfide bond formation and a subsequent disulfide bond-independent phase (Waring, 2000). Additionally, the α *methyl dopa resistant* (*amd*) gene product, which acts in the conversion of dopamine during the polymerization of the adult cuticle, is required in the follicle cells for proper vitelline membrane crosslinking (Konrad et al., 1993). This suggests that a similar set of dopamine conversion reactions catalyzed by Yellow-g and Yellow-g2 may be necessary for the crosslinking of the vitelline membrane just prior to egg laying. Consistent with this hypothesis, we observed that eggs laid by homozygous *yellow-g* mutant females are highly sensitive to sodium hypochlorite (bleach), and the majority of these embryos burst upon brief exposure (data not shown). Of the remaining, intact embryos, 100% were permeable to the dye neutral red (data not shown, performed as described [LeMosy and Hashimoto, 2000]), which has been used to assay vitelline membrane defects (Degelmann et al., 1990; Komitopoulou et al., 1983; Konrad et al., 1993). These results are indicative of a failure to crosslink the vitelline membrane and further implicate *yellow-g* in the crosslinking process. However, this hypothesis does not explain the specific expression of the *yellow-g* and *yellow-g2* genes in the follicle cells producing the micropyle late in egg chamber development. It is possible that crosslinking of the vitelline membrane or inner chorion layer within this specialized structure requires distinct

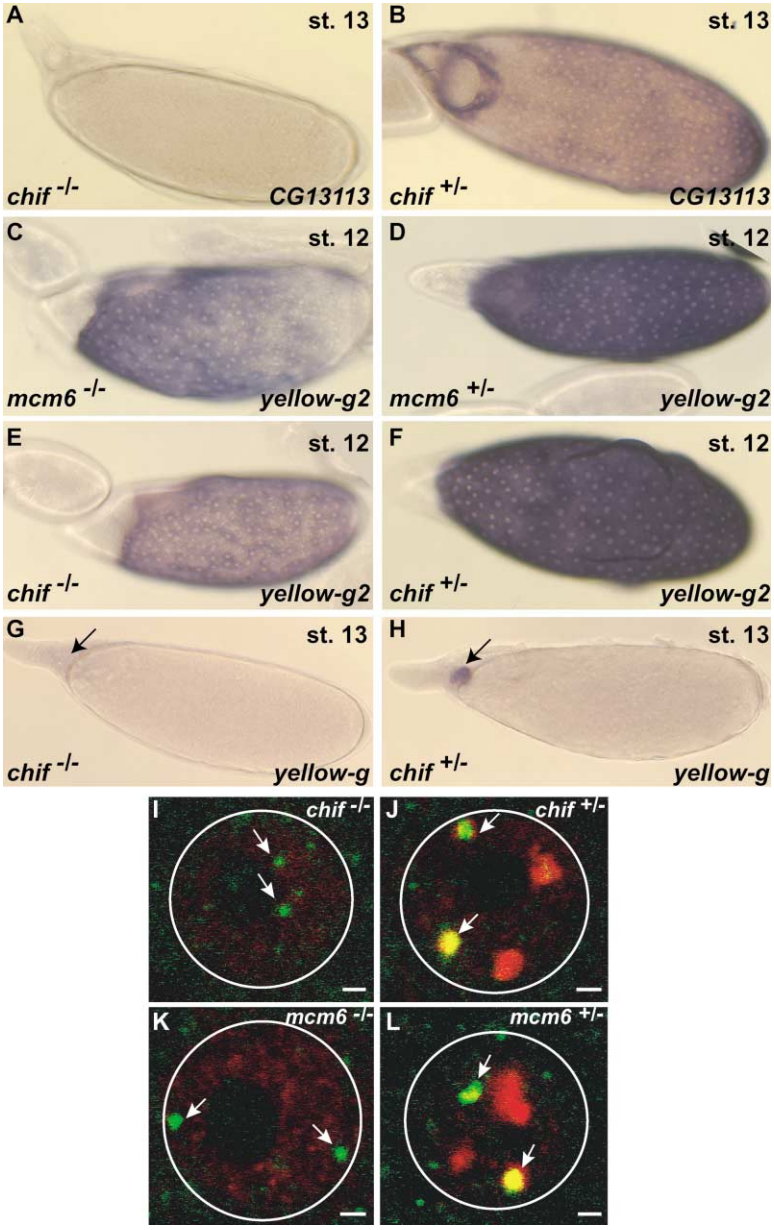

Figure 5. Amplified Genes Are Poorly Expressed in Replication Factor Mutants that Result in Decreased Amplification

RNA in situ hybridization for *CG13113* indicates that transcript levels are reduced in the *chiffon* female-sterile mutant ([A], genotype: $chif^{QW16}/chif^{WD18}$) as compared to heterozygous siblings with one wild-type copy of *chiffon* ([B], genotype: $chif^{QW16}/TM3$ or $chif^{WD18}/TM3$). RNA in situ hybridization for *yellow-g2* shows that transcript levels are reduced in the *mcm6* female-sterile mutant ([C], genotype: $mcm6^{fs(1)k1214}/mcm6^{fs(1)k1214}$), compared to heterozygous siblings ([D], genotype: $mcm6^{fs(1)k1214}/FM6$). *yellow-g2* (E) and *yellow-g* ([G], arrow) transcript levels are reduced in the *chiffon* female-sterile mutant (genotype: $chif^{QW16}/chif^{WD18}$), compared to heterozygous siblings ([F and H], genotype: $chif^{QW16}/TM3$ or $chif^{WD18}/TM3$). Anterior is left.

FISH to DAFC-30B and -62D concurrently (green, arrows) with BrdU colabeling (red) in *chiffon* mutant egg chambers shows that DAFC-30B and -62D are not amplified to a significant extent ([I], genotype: $chif^{QW16}/chif^{WD18}$), as compared with heterozygous siblings ([J], genotype: $chif^{QW16}/TM3$ or $chif^{WD18}/TM3$). Similar results were observed for the *mcm6* mutant females ([K], genotype: $mcm6^{fs(1)k1214}/mcm6^{fs(1)k1214}$) and their sibling controls ([L], genotype: $mcm6^{fs(1)k1214}/FM6$). A single follicle cell nucleus is shown for each. The scale bar represents 1 μm.

regulation or timing. A more detailed analysis of the eggshell defect and biochemical studies of Yellow-g and Yellow-g2 will help us to better understand the steps necessary for vitelline membrane crosslinking and will uncover any specialized micropyle functions.

DAFC-30B and DAFC-62D provide insights into the use of amplification as a developmental strategy. All of the previously characterized amplified genes play a purely structural role in eggshell formation; no enzymes necessary for proper eggshell formation have been examined. None of the genes of DAFC-30B and DAFC-62D encode known structural components of the eggshell. However, several of the amplified genes that are highly expressed in follicle cells, including *CG18419* and the *yellow-g* genes, encode products predicted to possess enzymatic, signal transduction, or transporting activities. Furthermore, at least *yellow-g* is essential for proper egg formation, thus revealing an additional function of amplification: to increase the levels of enzymes needed to catalyze developmentally important reactions. Thus the identification of additional amplicons highlights genes likely to be crucial in developmental events and opens the possibility that other tissues employ amplification to maximize gene expression during differentiation. It is surprising that a 4- to 6-fold increase in gene copy number would affect gene product levels in a developmentally significant manner. It is possible, however, that copy number increases are considerably higher in subsets of follicle cells, or that the replication process itself facilitates transcription.

The follicle cell amplicons serve as superb model metazoan replicons, permitting delineation of *cis*-regulatory elements, identification of replication proteins, and clarifying the developmental control of the initiation

and elongation. Developmental distinctions between DAFC-62D and the previously studied DAFCs provide clues into how origin firing can be linked to developmental signals. Previously, we showed by real-time PCR that replication initiates at DAFC-66D and -7F, coupled with replication fork movement, during egg chamber stages 10B and 11. Subsequently (stages 12 and 13), origins cease firing and only existing replication forks move bidirectionally to produce a gradient of copy number that extends over 100 kb (Claycomb et al., 2002). Furthermore, the replication initiation factor ORC2 only localizes to amplification origins during the initiation phase and dissociates at the onset of the elongation phase. Replication factors involved in multiple steps of DNA replication, such as MCM2-7 and PCNA, colocalize with BrdU throughout amplification (Claycomb et al., 2002; Royzman et al., 1999; Spradling, 1981; Whittaker et al., 2000).

DAFC-62D behaves differently from these amplicons and from DAFC-30B. There is a final increase in copy number at a very precise region of the amplicon, about 1.5 kb downstream of *yellow-g2*, during stage 13. As it is the peak of amplification, this region is likely to possess a replication origin. Understanding how DAFC-62D can undergo a final initiation hours after ORC is no longer detectable at origins by immunofluorescence will provide insights into the control of replication initiation. The additional replication in stage 13 may occur in only subsets of follicle cells, and ORC could persist specifically at DAFC-62D in these cells. For example, additional gene copies could permit optimal levels of expression of the *yellow-g* genes in the follicle cells building the micropyle.

We initiated these studies to devise a systematic approach for finding developmental amplicons. We have demonstrated that the microarray assay is sensitive and can detect low levels of gene amplification, and we have shown that amplification levels as low as 4-fold can be developmentally important. Thus, we believe our approach will be invaluable in surveying for gene amplification in a number of tissues and in a variety of organisms where amplification has not been detected. Not only has the microarray strategy identified additional amplicons, but when coupled with the power of a genetic organism, it has proven to be a functional genomics approach for highlighting genes involved in specific developmental pathways.

Experimental Procedures

Quantitation of DNA Copy Number on Microarrays

Drosophila Gene Collection strains were grown in deep-well 96-well plates with 1 ml media. Plasmid minipreps were done using Millipore MultiScreen and yields quantitated using a Tecan GENios microplate fluorometer and Picogreen (Molecular Probes). The cDNA inserts were PCR-amplified, and primer sets used are available upon request. PCR products were isopropanol precipitated, analyzed to be the predicted size and to have an average concentration of 370 μg/ml. Microarrays were printed using a Cartesian Technologies arrayer on Corning (experiment 1) or Ultragap slides (experiments 2 and 3) and crosslinked with a 2400UV Stratalinker at 300 mJ.

Genomic DNA was isolated from embryos or FACS sorted 16C follicle cells from a *y; cn bw sp* stock as described previously (Lilly and Spradling, 1996). 200 ng of each was digested with RsaI and labeled by random priming with either Cy3 or Cy5-dCTP (Pollack et al., 1999) and hybridized to the slides in 3.4X SSC, 0.3% SDS with 30 μg human Cot 1 DNA and 100 μg yeast tRNA in 50 μl. The hybridization was at 55°C overnight, and slides were washed in 0.1X SSC at room temperature.

Fluorescent hybridization was detected on an Axon Instruments GenePix 4000A microarray scanner, with manual adjustment of the scan area for each feature. Spots having an intensity less than 100 were discarded. The raw ratios (16C follicle cell/embryo) were calculated using the background substracted median intensities of the remaining features. The ratios were normalized by dividing each raw ratio by the mean raw ratio. Clones with a ratio higher than two standard deviations from the mean were scored as significantly amplified.

In the first experiment, 3643 of the clones were scored. The significance cut off was an amplification level of 2.1 or higher, and 13 clones were significantly amplified. In the second experiment, 5568 clones were scored. 63 had ratios higher than the cut off amplification value of 1.8 or higher. In the third experiment, 5929 clones were scored. These were done in duplicate on the same slides; Table 1 shows the average values. 28 clones showed amplification values of 1.8 or higher, the significance cut off in this experiment.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed as described (Claycomb et al., 2002) except that ABI SYBR Green Master Mix was used (Applied Biosystems) and primers in 5 kb intervals across 30B10 and 62D5, and in 2 kb intervals across the 62D5 amplification peak were supplied by IDT and Genelink. Primer sequences are available upon request. All experimental PCR reactions at the amplicons were compared to nonamplified control loci on the same chromosome arm to calculate fold amplification. For DAFC-30B, the nonamplified control locus was located at 30B2, and for DAFC-62D, the nonamplified control locus was located at 62C5. The relative fluorescence of control loci was initially compared to values obtained with the nonamplified control *ry* primer set (Claycomb et al., 2002), to assure that they were valid nonamplified loci.

Fluorescent In Situ Hybridization, BrdU Labeling, and Confocal Microscopy

Ovaries were dissected, labeled with 4 μg/ml BrdU, and prepared as described (Calvi et al., 1998; Royzman et al., 1999). BrdU was detected with donkey anti-mouse Rhodamine-RedX (Jackson Immunoresearch) at 1:250. After BrdU detection, ovaries were refixed (Royzman et al., 1999), and FISH was performed as described (Calvi et al., 1998). The probes used were generated from 10 kb PCR products (Clontech Advantage 2 PCR Kit, BD Biosciences). The DAFC-30B probe spans genes *CG18419* to *CG31883*, and the DAFC-62D probe covers from *CG5714* to the intergenic region between the *yellow-g* genes. Primers are available upon request.

Templates for the PCR were BACR07D23 for DAFC-30B and BACR22J16 for DAFC-62D (BAC PAC/CHORI). FISH probes were detected with goat anti-DIG FITC at 1:200 (Enzo), and samples were mounted in Vectashield (Vector Labs) or Slowfade (Molecular Probes). All images were collected on a Zeiss Axiovert 100M Meta confocal microscope with LSM51 Software using a 100× Plan Aprochromat objective and the filters set according to the manufacturer's parameters.

RNA In Situ Hybridization

Templates for in situ probes were generated by PCR of each gene's largest predicted exon from *Oregon-R* genomic DNA. Primers used were 30–35-mers and added a 5′ EcoRI restriction site or a 3′ XhoI site to the PCR product. Primers were supplied by Genelink. Sequences are available upon request. PCR products were purified with the Qiagen PCR Clean-up kit, digested with EcoRI and XhoI (NEB), and then cloned into pBluescript SK+ using T4 DNA ligase (NEB). Sense and antisense probes were made as described (Royzman et al., 1997), and hybridizations were done at 55°C on ovaries as described (Royzman et al., 2002). The images in Figures 3C, 3D, 3H, and 3K were captured using the 25× Zeiss Neofluar objective, water immersion. A Plan Neofluar 20× objective was used for all others. A Zeiss Axiophot microscope with a SPOT RT CCD camera and software was used to capture all images.

yellow-g Mutant Analysis and Scanning Electron Microscopy

The line *EY01493* contains an EPgy2 P element in the 3′ exon of *yellow-g*. The line was generated by the P-element Screen/Gene Disruption Project of the Bellen/Rubin/Spradling labs (Bellen, 2003; Spradling et al., 1999) and obtained from the Bloomington Stock Center (#15512). The deficiency, *Df(3L)Aprt32/TM6 Ubx e*, removes the 62B1-62E3 region and was obtained from the Bloomington Stock Center (#5411) (Wang et al., 1994).

The EPgy2 line was crossed to the deficiency line, and the progeny were collected for egg laying experiments. Heterozygous sibling females were separated from mutant *EPgy2/Df* females, and egg laying was monitored over 6 to 12 hr intervals. Fertility was determined by allowing the females to lay eggs for 3 days and monitoring for larvae.

SEM was performed on a Jeol JSM5600LV SEM in low vacuum mode with an acceleration voltage of 5 kV and a spot size of 42. Images were collected using the shadow mode of the backscatter detector. Samples were prepared by adhering 0–12 hr embryos on double-stick carbon tabs.

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